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Characterization of phenanthrene degradation by strain *Polyporus* sp. S133

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Abstract

Polyporus sp. S133, a fungus collected from contaminated soil, was used to degrade phenanthrene, a polycyclic aromatic hydrocarbon, in a mineral salt broth liquid culture. A maximal degradation rate (92%) was obtained when *Polyporus* sp. S133 was cultured for 30 days with agitation at 120 r/min, as compared to 44% degradation in non-agitated cultures. Furthermore, the degradation was affected by the addition of surfactants. Tween 80 was the most suitable surfactant for the degradation of phenanthrene by *Polyporus* sp. S133. The degradation rate increased as the amount of Tween 80 added increased. The rate in agitated cultures was about 2 times that in non-agitated cultures. The mechanism of degradation was determined through the identification of metabolites; 9,10-phenanthrenequinone, 2,2'-diphenic acid, phthalic acid, and protocatechuic acid. Several enzymes (manganese peroxidase, lignin peroxidase, laccase, 1,2-dioxygenase and 2,3-dioxygenase) produced by *Polyporus* sp. S133 were detected during the incubation. The highest level of activity was shown by 1,2-dioxygenase (187.4 U/L) after 20 days of culture.

Key words: phenanthrene; biodegradation; metabolites; *Polyporus* sp. S133
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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants having toxic, genotoxic, mutagenic, and carcinogenic properties (Mastrangela et al., 1997). These compounds are universal products of organic matter combustion and are prevalent in the petroleum industry, in the coal-refining process, in automobile exhaust fumes, and after forest fires (Blumer, 1976; Gisalba, 1983). Consequently, the environment has become highly polluted with different PAHs. Phenanthrene, a three-ring angular PAH, is known to be a skin photosensitizer and mild allergen (Fawell and Hunt, 1998; Hwang and Cutright, 2002). Biodegradation is one of the primary means of eliminating phenanthrene from contaminated sites. However, the biodegradation of phenanthrene is restricted by a limited bioavailability (Thomas et al., 1986; Barr and Aust, 1994). The bioavailability of phenanthrene in liquid medium may be increased by the application of surfactants. Some surfactants increase the bioavailability of organic contaminants through solubilization into the hydrophobic core of micelles in solution. This has been observed for surfactants in excess of their critical micelle concentration (Wilson and Jones, 1993).

During the past decade, a variety of microorganisms capable of catabolizing phenanthrene as a source of carbon and energy have been recognized (Balashova et al., 1999; Churchill et al., 1999; Da Silva et al., 2004). Notably, various white rot fungi seem to be involved in the degradation of PAHs containing more than two rings. However, degradation pathways for phenanthrene's catabolism by *Polyporus* species are not well understood. Under mesophilic conditions, white rot fungi species metabolize phenanthrene at different sites of the molecule, presumably via ligninolytic enzymes and dioxygenase on the aromatic nucleus. The initial attack in the K-region, presumably by a ligninolytic enzyme, leads to the formation of 9,10-phenanthrene quinone (Hammel et al., 1992). However, further degradation products of 9,10-phenanthrene quinone have not been elucidated by using white rot fungi. The objective of the present study is to investigate the capability of fungi screened from nature to degrade phenanthrene in liquid medium. The production of enzymes and metabolites during the degradation process was also investigated to evaluate the availability of fungi for bioremediation in soil.

1 Materials and methods

1.1 Microorganism and culture conditions

Polyporus sp. S133 isolated from a petroleum-contaminated soil in Matsuyama City, Ehime, Japan, was used for experimentation. The fungus was maintained

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on malt extract agar (2% (*W/V*) malt extract, 2% (*W/V*) glucose, 0.1% (*W/V*) polypeptone, and 1.5% (*W/V*) agar) in a plastic Petri dish at 4°C prior to use. *Polyporus* sp. S133 was selected based on its ability to degrade phenanthrene in a solid agar medium containing 20 mL malt extract agar with the addition of phenanthrene dissolved in dimethylformamyde (DMF) and 300 mg/L chloramphenicol or benomill to inhibit bacterial growth, then incubated at room temperature for two weeks and observed daily. A single colony of phenanthrene-degrading fungus was transferred to a mineral salt broth medium containing phenanthrene. The mineral salt broth (MSB) medium contained in distilled water (in g/L, pH 5.6): glucose 10, KH_2PO_4 2, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2{\cdot}2H_2O$ 0.1, ammonium tartrate 0.2, and trace elements 10 mL. The trace element solution comprised (mg/L): $FeSO_4{\cdot}7H_2O$ 12, $MnSO_4{\cdot}7H_2O$ 3, $ZnSO_4{\cdot}7H_2O$ 3, $CoSO_4{\cdot}7H_2O$ 1, and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 1 (Arora and Gill, 2001). The fungal inoculum was prepared by growing fungus on malt extract agar plates at 25°C for 7 days. The inoculum was added to a flask containing the mineral salt broth medium.

1.2 Chemicals and regents

Phenanthrene, 2,2'-diphenic acid, phthalic acid, and protocatechuic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Malt extract and polypeptone were purchased from Difco, USA. Thin layer chromatography (TLC) aluminium sheets (Silica gel 60 F_{254}, 20 cm × 20 cm) were obtained from Merk, Germany. The silica gel used for column chromatography (wakogel S-1), and all other chemicals were purchased from Wako Pure Chemical Industry Co., Ltd., Japan. 9,10-Phenanthrene quinone was prepared from phenanthrene by chromate oxidation (Vogel, 1989). Phenanthrene was dissolved in sulfuric acid that was saturated in $Na_2Cr_2O_7$. The solution was heated to 90–95°C in a water bath and purified phenanthrene and potassium dichromate were added. The solution was then heated in a boiling water bath for 30 min. 9,10-Phenanthrene quinone was filtered with suction and washed with water until free from chromium salt. The concentrated, crude 9,10-phenanthrene quinone, obtained in about 60% yield, was purified by column chromatography.

1.3 Biodegradation assays

Experiments were performed in 100-mL Erlenmeyer flasks containing 20 mL of liquid medium plus 1 mmol/L phenanthrene dissolved in DMF to 1 mL. As the strains have different growth rates, the period of incubation was varied from day 5 to day 7 to obtain a similar radial growth and to minimize the variation in starting inoculums. Mycelial plugs of a selected fungus were cut from the outer edge of an actively growing culture on an inoculum plate. Three 5-mm disks obtained by punching out with a cork-borer from the outer edge of an actively growing culture of a particular fungus were inoculated into a 100-mL Erlenmeyer flask containing 20 mL of MSB medium supplemented with 1 mmol/L of substrate. The flasks were incubated at 25°C. Growth and substrate consumption were determined at 7-day intervals. One set of inoculated flasks was incubated stationary. The effect of varying the surfactant on phenanthrene degradation was studied using 0.5% Tween 80 and 0.5% Tween 20. The effect of varying the agitation rate (80, 120, and 160 r/min) and Tween 80 concentrations (0.1%, 0.5%, and 1%) were also studied. All media were sterilized by autoclaving at 121°C for 20 min. Control experiments were performed by incubating phenanthrene in autoclaved cultures (121°C for 20 min) and by incubating MSB medium with phenanthrene without an inoculum. All assays were conducted in triplicates. Before the incubation, a flask was selected for immediate extraction. All remaining flasks were incubated for 15 and 30 days.

1.4 Analytical methods

After incubation, the culture broth was blended with ethyl acetate and acidified with 1 mol/L HCl. The liquid medium and fungal bodies were separated by filtration, and the liquid medium and fungal bodies were extracted with ethyl acetate, respectively. Each extract was combined and purified by column chromatography using dichloromethane:methanol (19:1) (200 mL). With this method, all phenanthrene initially present in the liquid medium were recovered. The extracts were concentrated and analyzed by gas chromatography-mass spectrometer (GC/MS) (QP-5050, Shimadzu, Japan). The amount of substrate was determined using 4-chlorobiphenyl as an internal standard. GC/MS was performed with a column 30 m in length and 0.25 mm in diameter, and a helium pressure of 100 kPa. The temperature was initially 80°C, held for 2 min, raised from 80 to 200°C at 20°C/min, then to 260°C at 7.5°C/min, and held for 4 min. The flow rate was 1.5 mL/min, interface temperature was 260°C, and injection volume was 1 μL. Degree of degradation was determined by comparison of the remaining phenanthrene between control and samples.

For detection of metabolites, MSB medium was prepared as described above. After inoculation of the medium with *Polyporus* sp. S133, the culture was pre-incubated by standing it for 7 days at 25°C in the dark for optimum growth. Phenanthrene dissolved in 100 μL of DMF and 10 μL of Tween 80 (1% solution) were added to the culture medium as described above. The incubation was conducted for 1–30 day at 25°C in the dark. The products of phenanthrene degradation in culture extracts were analyzed by TLC on silica gel 60 F_{254} (20 cm × 20 cm, thickness 0.25 mm) using hexane:chloroform (20:10, *V/V*) as the solvent system for short-term incubation, and dichloromethane:ethyl acetate:methanol (20:30:10, *V/V/V*) as the solvent system for long-term incubation. The locations of the compounds on TLC plates were detected using UV light. The R_f values of the detected spots were compared with those of authentic compounds known or suspected to be metabolites of phenanthrene degradation. UV-Vis absorption spectra were recorded on a UV-Vis spectrophotometer (Shimadzu 1600, Japan). The metabolites were identified by comparing their retention time, mass spectra and absorption spectra with those of the

corresponding authentic standards.

Authentic phenanthrene, 2,2'-diphenic acid, phthalic acid, and protocatechuic acid were used as standards. The extracts were purified using silica gel column chromatography by successive elution with several solvent combinations. The metabolites were tentatively identified by comparing R_f values and UV properties (i.e., quenching under a short wavelength ($UV_{254\ nm}$) or blue-green fluorescence under a long wavelength ($UV_{365\ nm}$)) of the samples to those of authentic compounds. As three authentic compounds viz 2,2'-diphenic acid, phthalic acid, and protocatechuic acid, could not be detected directly by GC/MS, an analytical derivatization procedure was used to detect these compounds with GC/MS; these compounds were subjected to trimethylsilylation (TMS). Similarly, extracts from phenanthrene-grown cultures were also derivatized and subjected to test the presence of these three compounds. After the vacuum drying of each eluate from phenanthrene-grown cultures (100 μL) in a vial, N,O-bis-trimethylsilyl acetamide (40 μL), pyridine (40 μL), and trimethylchlorosilane (20 μL) were added. Trimethylsilylation of the eluate was conducted for 10 min at 80°C without contact with moisture. The trimethylsilyl derivatives of the extract were analyzed by GC (Shimadzu GC-17) equipped with a TC-1 capillary column (30 m × 0.25 mm × 0.25 μm) using a gradient of 60°C for 2 min, raised to 150°C at 15°C/min, then raised to 300°C at 25°C/min, and maintained at 300°C for 6 min. Injector and interface temperatures were 260°C. In order to confirm the metabolites of phenanthrene's degradation and to determine the degradation pathway, a Gas chromatograph-mass spectrophotometer (QP5050, Shimadzu, Japan) was used. The conditions for GC/MS consisted of the use of a detector at 1.3 eV, scan intervals of 1 sec, and a mass range of 50–500. The mass spectra of individual total ion peaks were identified by comparison with the Wiley7 mass spectra database. Mass profiles were also compared with the spectra of authentic standards which were analyzed similarly.

1.5 Enzyme assays

Extracellular enzyme production was investigated in the MSB medium. After incubated for 5, 10, 15, 20, 25, and 30 days, the samples were homogenized at 10,000 r/min and the enzymatic activities in the crude supernatant were determined using an UV-Vis spectrophotometer. Manganese peroxidase activity was assayed using 50 mmol/L malonate buffer and dimethoxyphenol in 20 mmol/L $MnSO_4$ (Wariishi et al., 1992). Laccase activity was assayed using syringaldazine in 100 mmol/L sodium acetate buffer (Tien and Kirk, 1984). Lignin peroxidase activity was determined using veratryl alcohol as a substrate (Kuwabara et al., 1984). 1,2-Dioxygenase and 2,3-dioxygenase were measured by a modified method (Nakazawa and Nakazawa, 1970). 1,2-Dioxygenase and 2,3-dioxygenase activities were assayed using catechol as a substrate. One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute and the activity was expressed in U/L.

2 Results and discussion

2.1 Degradation of phenanthrene by selected fungi

The rate of degradation was above 40% for 30 days incubation with *Polyporus* sp. S133. *Polyporus* sp. S133 degraded 44% of phenanthrene at 1 mmol/L on day 30. It was observed that by day 30 and in the stationary cultures, *Polyporus* sp. S133 formed filamentous mats on the surface of the growth medium, while in the set incubated with agitation, uniform pellets were formed. Figure 1 shows 92% degradation was achieved at 120 r/min on day 30, as compared to 44% degradation in the stationary cultures. The increased efficiency of degradation could be due to the physiological state of the fungus as pellets and increased mass transfer between the cells and the medium. Biodegradation by white rot fungi has been attributed to the extracellular activity of oxidative enzymes such as laccase. Laccase production was greatest in agitated cultures and hence maximum degradation was achieved. The oxygen concentration is directly dependent on the air flow rate. Stirring increases the contact between the reagents (substrate, oxygen, and biomass), thus enhancing mass transfer and, as a consequence, the biodegradation rate (Collina et al., 2005). In stationary cultures, the formation of a mat at the surface restricts the transfer of oxygen to the cells beneath the surface and in the medium resulting in oxygen limitation, which inhibits the oxidative enzymes and prevents the degradation.

Figure 1 also shows the effect of surfactants on the degradation of phenanthrene. The highest degradation rate was obtained with Tween 80 (72%) after 30-day incubation. When the present cultures were supplemented with Tween 80, degradation rates were about 1.5-fold higher than those obtained in control cultures (without Tween 80). Furthermore, enzymatic activity levels were maintained throughout the culture, which might be correlated with greater stability of isoenzyme produced. One of the benefits of using surfactants like Tween 80 is a better dissolution of very hydrophobic substrates (Kapich et al., 1999). On the other hand, Tween 80 could promote both the uptake and release of compounds from the cells through modification of plasma membrane permeability. Moreover, Tween 80 increases the solubility of petroleum components such as phenanthrene, or lowers the interfacial

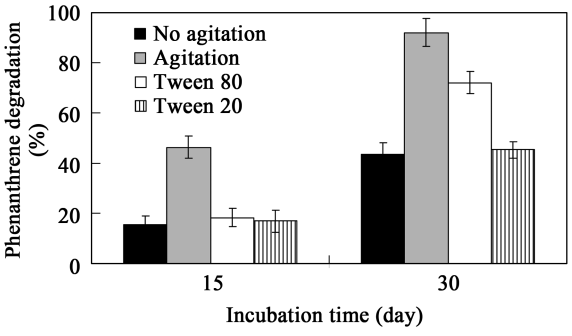

Fig. 1 Effect of agitation and kind of surfactant on degradation of phenanthrene by *Polyporus* sp. S133.

tension to enhance the mobility of petroleum. Typical desirable properties include solubility enhancement, surface tension reduction, critical micelle concentrations, wetability, and foaming capacity (Asther et al., 1987).

2.2 Effect of surfactant concentration on the degradation of phenanthrene and the growth of fungi

Figure 2a shows the effect of surfactant concentrations. *Polyporus* sp. S133 degraded 30%, 47%, 72%, and 61% of phenanthrene without surfactant, and with 0.1%, 0.5%, and 1.0% Tween 80 on day 30, respectively. Of all the concentrations of surfactant tested, the lowest rate of degradation of phenanthrene was obtained with no surfactant (30%). At 0.1% Tween 80, the rate of degradation on day 30 was only 47%. Among all the concentrations tested, 0.5% Tween 80 was the best with 72% degradation. The degradation rate decreased 1.0% (61%) while the concentration of Tween 80 was increased. Due to their chemical features, surfactant molecules may sorb directly onto solid surfaces or may interact with sorbed surfactant molecules. The concentration of surfactant may affect the growth of fungi. The sorption mechanism is dependent on the nature of the sorbent and the concentration of surfactant. At a low concentration, the surfactant molecule may be sorbed on a mineral surface that has very few sorbed surfactant molecules, and sorption may occur mainly due to Van der Walls interaction between the hydrophobic and hydrophilic moieties of the surfactant and the surface (Adeel and Luthy, 1995; Brownawell et al., 1997; Fytianos et al., 1998; Ou et al., 1996). Moreover, the sorption of a nonionic surfactant such as Tween 80 reaches a maximum on a solid surface when the solution is near or just at the critical micelle concentration of the surfactant (Adeel and Luthy, 1995; Kibey and Hayes, 2001).

Figure 2b shows the effect of surfactant concentration on the growth of *Polyporus* sp. S133 in agar medium contaminated with phenanthrene. It is clear that in the presence of Tween 80 (0.5%), the growth of fungi increased rapidly. At the end of the incubation (7 day), *Polyporus* sp. S133 covered 88% of the Petri disk with Tween 80 compared to 56% without Tween 80. These results show that the biodegradation activity of phenanthrene-degrading fungi is influenced by the bioavailability of phenanthrene in aqueous phase. By increasing the solubility of solid phenanthrene, Tween 80 facilitates the transport of this organic substrate to microbial cells and enhances the metabolism of this hydrocarbon. This positive effect on the growth rate is not a general response to the addition of surfactant since some surfactants may also inhibit the growth and biodegradation (Laha and Luthy, 1992; Stelmack et al., 1999).

2.3 Effect of agitation rate on phenanthrene degradation

Figure 3 shows the effect of agitation rate on the degradation of phenanthrene by *Polyporus* sp S133. Of all the measured variables, the lowest rate of degradation was observed in non-agitated cultures. This is probably caused by poor oxygenation, despite the control of the dissolved oxygen concentration. The large size of the pellets found in non-agitated cultures could also be responsible, by rendering the diffusion of enzymes out of the pellets more difficult. Among the agitation rates tested, 120 r/min was the best with 92% degradation. The degradation rate decreased while the agitation rate was increased to 160 r/min (55%). At 160 r/min, the production of enzymes was low and the extracellular protein concentration in the culture was also at its lowest. The low levels of activity might be caused by the destruction of the enzymes by shear stress. The major cause of the lower degradation rate at a high agitation rate must be a lower production rate, caused by the shear stress (Lejune and Baron, 1995). Furthermore, agitation has two main effects on the cultivation of fungi. First, it is important to ensure the supply of nutrients, especially oxygen. Good mixing, mass and heat transfer require a threshold level of agitation. On the other hand, a high agitation rate leads to a high energy dissipation rate connected with high shear stress, which may result in the fragmentation and damage of cells and the mycelial network (Amanullah et al., 2000; Li et al., 2000; Mittard and Riba, 1988; Nielsen et al., 1995).

2.4 Enzymatic activity of selected fungi

Several enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP), laccase, 1,2-dioxygenase and 2,3-dioxygenase were detected in the culture produced by *Polyporus* sp. S133. The levels of MnP, LiP, and laccase activity were the highest after 15 days of cultivation (69.7, 54.3 and 77.6 U/L, respectively) while 1,2- and 2,3-dioxygenase showed their highest levels after 20 days

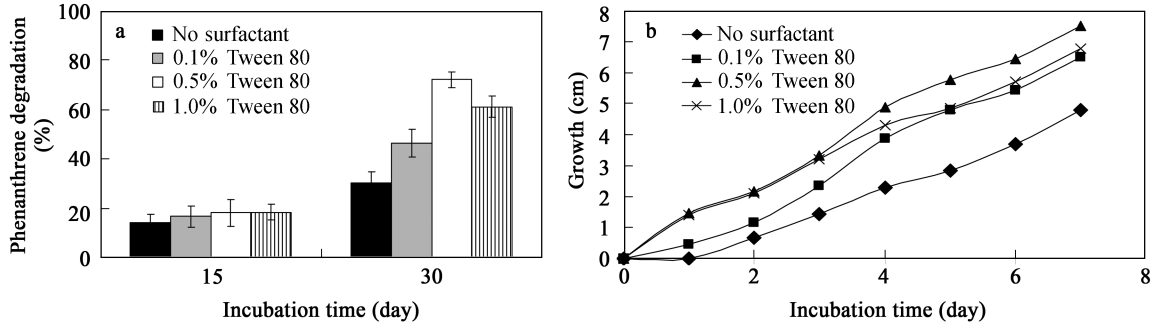

Fig. 2 Effect of Tween 80 concentration on degradation of phenanthrene by *Polyporus* sp. S133 in liquid medium (a), and fungal growth on agar medium (b).

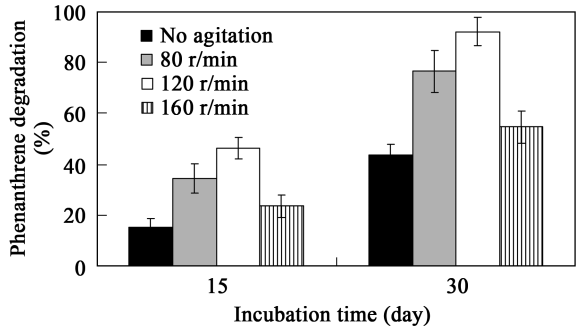

Fig. 3 Effect of agitation rate on degradation of phenanthrene by *Polyporus* sp. S133.

(187.4 and 23.2 U/L, respectively) (Fig. 4). Those ligninolytic and dioxygenase enzymes play an important role in the oxidization of various environmental pollutants such as chlorophenol, aromatic dyes, and polycyclic aromatic hydrocarbons (Mester and Tien, 2000). LiP is able to oxidize various aromatic compounds, while MnP oxidizes almost exclusively Mn(II) to Mn(III), which then degrades phenolic compounds (Mester and Tien, 2000). Laccase is a copper-containing oxidase that reduces molecular oxygen to water and oxidizes phenolic compounds (Wasenberg et al., 2003). In most species, peroxidase and laccases are expressed as several isoenzymes. Both types of ligninolytic enzymes are glycosylated, which may increase their stability (Nie et al., 1999). Dioxygenase is the enzyme in bacteria that degrades PAHs (Pinyakong et al., 2000). Dioxygenase initially attacked the PAHs at both the 1,2-position and the 2,3-position.

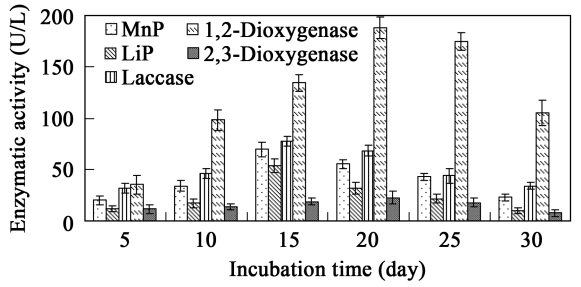

Fig. 4 Change in enzymatic activity during incubation of *Polyporus* sp. S133.

2.5 Identification of metabolites

Four metabolites were detected during the degradation of phenanthrene by *Polyporus* sp. S133 (Table 1). The TLC and UV-Vis spectrophotometric analyses were initially performed to indicate the presence of different intermediates in the degradative pathway by combining short- and long-term incubation extracts of the phenanthrene-grown culture. GC/MS and MS were carried out to conclusively prove the presence of these intermediates using extracts of short- and long-term incubations separately. The identity of four of these metabolites was confirmed using authentic standards. One metabolite (I) having a R_f value of 0.44, gave a UV spectrum with λ_{max} of 282, 346, and 416 nm, similar to that of synthesized 9,10-phenanthrene quinone. The spectrum of compound I (*m/z* 208, M^+), which had a retention time (t_R) of 13.4 min, is shown in Fig. 5a. The GC retention time, MS properties of the M^+ at *m/z* 208, and the significant fragment ions at *m/z* 152 and 180 (M^+-28), corresponding to the respective sequential losses of –CO, were identical to those of synthesized 9,10-phenanthrene quinone. Another metabolite (II) with a R_f value of 0.38, gave a UV spectrum with λ_{max} of 225 and 285 nm, similar to that of the authentic 2,2'-diphenic acid standard. GC/MS of compound II (Fig. 5b) with a GC retention time of 14.7 min indicated a di-TMS compound. MS of the 2,2'-diphenic acid produced from phenanthrene gave an apparent molecular ion M^+ at *m/z* 386 for TMS-derivatives and significant fragment ions at *m/z* 371 (M^+-15), sequential loss of methyl (–CH_3), and 269 (M^+-117), sequential loss of –$COOSi(CH_3)_3$, as well as the expected fragment ions at 147, 117 and 73 [$(CH_3)_3Si$].

Polyporus sp. S133 degraded phenanthrene via 2,2'-diphenic acid to phthalic acid and protocatechuic acid in a similar incubation time. When the short-term extract was subjected to GC/MS, only 9,10-phenanthrene quinone and 2,2'-diphenic acid could be identified whose retention time and mass-fragmentation pattern matched those of the synthesized compound. However, when a long-term extract was analyzed, other peaks from the short-term extract

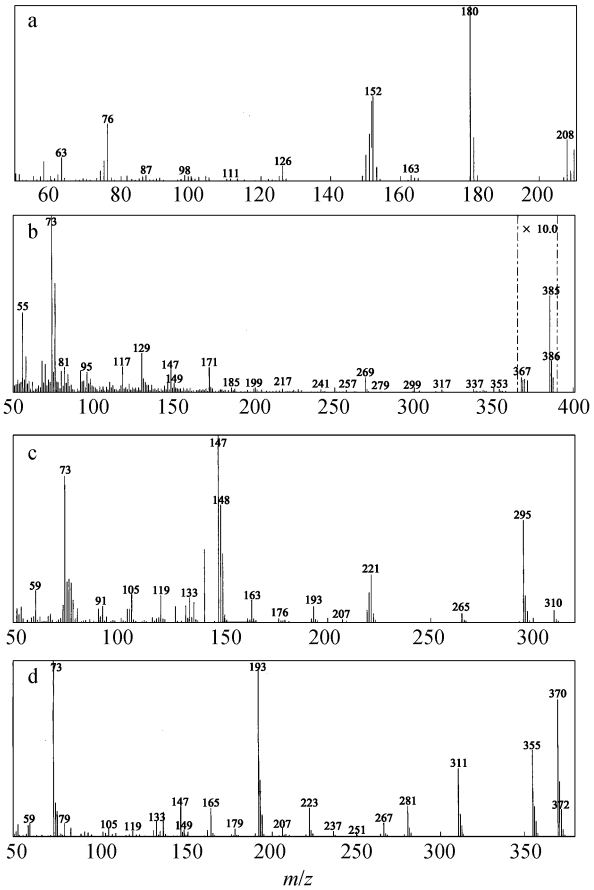

Fig. 5 Mass spectral profiles of phenanthrene metabolites: 9,10-phenanthrene quinone (a), 2,2'-diphenic acid-TMS derivative (b), phthalic acid-TMS derivative (c), and protocatechuic acid-TMS derivative (d).

Table 1 Mass spectral analysis of the principal metabolites detected during the degradation of phenanthrene by *Polyporus* sp. S133

Metabolite	Retention time (min)	*m/z* of fragment ions (% relative abundance)	Possible structure
I	13.4	208 (23, M^+), 76 (33), 150 (15), 151 (27), 152 (48), 180 (100), 181 (25)	9,10-Phenanthrene quinone (confirmed with a synthesized compound)
II	14.7	386 (3, M^+), 73 (100), 117 (14), 147 (18), 269 (11), 371 (1)	2,2'-Diphenic acid-TMS derivative (confirmed with a standard)
III	8.9	310 (6, M^+), 73 (79), 91 (9), 119 (14), 147 (100), 148 (63), 149 (37), 163 (12), 193 (8), 221 (25), 295 (54)	Phthalic acid-TMS derivative (confirmed with a standard)
IV	11.2	370 (75, M^+), 73 (100), 137 (14), 147 (17), 164 (16), 193 (97), 194 (32), 195 (12), 223 (16), 281 (17), 311 (38)	Protocatechuic acid-TMS derivative (confirmed with a standard)

disappeared and new peaks appeared, the retention time and fragmentation pattern of compound III and IV matching those of authentic phthalic acid and protocatechuic acid, respectively. No phenanthrene peak was apparent, probably because of the total degradation of phenanthrene.

TLC of the ethylacetate-extractable metabolites of phenanthrene showed one metabolite (III) having a R_f value of 0.2. This metabolite had the same R_f value and UV characteristics (λ_{max}, 226 and 275 nm) as the authentic phthalic acid standard. The mass spectrum of compound III (Fig. 5c), eluting at retention time 8.9 min, had a molecular ion at *m/z* 310 and fragmentation ions at *m/z* 295 (M^+-15), sequential loss of methyl (–CH_3), 221 (M^+-89), sequential loss of –$OSi(CH_3)_3$, and 193 (M^+-117), sequential loss of –$COOSi(CH_3)_3$, as well as the expected fragment ions at 163, 147, 119, 91, and 73 [$(CH_3)_3Si$].

The last metabolite (IV) with an R_f value of 0.53, gave an UV spectrum with λ_{max} of 205 and 283 nm, similar to that of the authentic protocatechuic acid standard. GC/MS of compound IV (Fig. 5d) with a GC retention time of 11.2 min indicated a tri-TMS compound. MS of the protocatechuic acid produced from phenanthrene gave an apparent molecular ion (M^+) at *m/z* 370 for TMS-derivatives and significant fragment ions at *m/z* 355 (M^+-15), sequential loss of methyl (–CH_3), and 281 (M^+-89), sequential loss of –$OSi(CH_3)_3$, as well as the expected fragment ions at 311, 193 and 73 [$(CH_3)_3Si$].

The pathway of phenanthrene degradation by *Polyporus* sp. S133 is proposed in Fig. 6. *Polyporus* sp. S133 grows in MSB medium with phenanthrene as a source of carbon and energy. Analysis of the culture extracts showed that *Polyporus* sp. S133 did not accumulate large quantities of aromatic metabolites during the cultivation period. Low levels of metabolite production by some PAH-degrading fungi may be related to the physical interaction between cell and hydrophobic substrates (Guerin and Jones, 1988). Based on the identification of various metabolites produced during the initial ring oxidation and ring cleavage processes, the metabolism of phenanthrene by *Polyporus* sp. S133 was explored.

These results showed that configuration of 9,10-oxidation and ring cleavage to give 9,10-phenanthrene quinone is the major fate of phenanthrene in ligninolytic *Polyporus* sp. S133. Phenanthrene metabolism in ligninolytic *Polyporus* sp. S133 differs from the pathway employed by most bacteria, which cleave this PAH between C_3 and C_4 (Gibson and Subramanian, 1984), and also differs from the process in nonligninolytic fungi

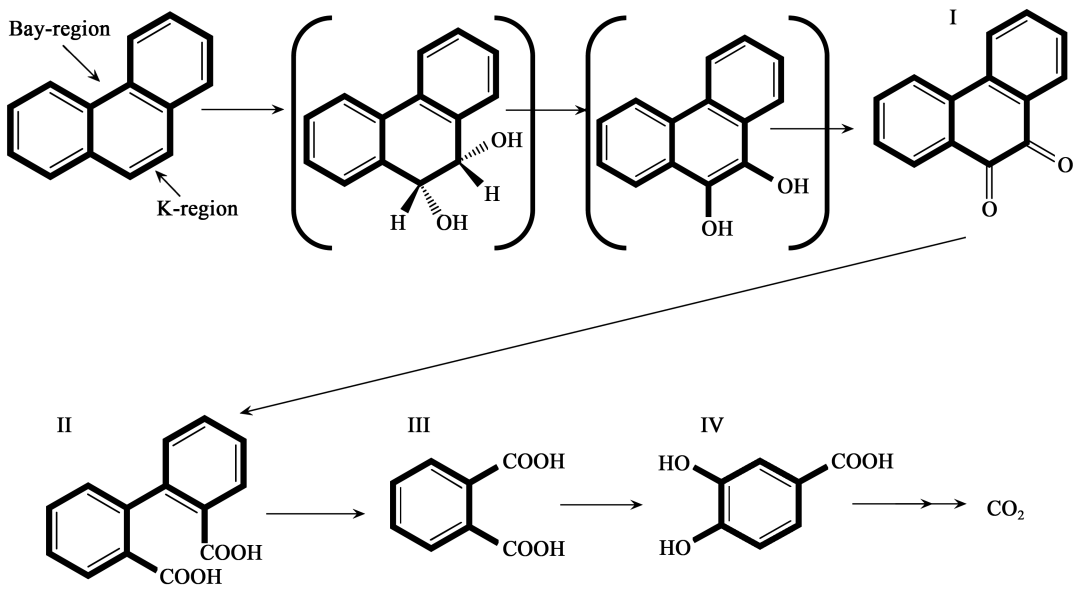

Fig. 6 A proposed pathway for the degradation of phenanthrene by *Polyporus* sp. S133. Compounds between brackets were not identified in our culture extracts.

and other eukaryotes, which are incapable of PAH ring fission (Gibson and Subramanian, 1984; Cerniglia and Yang, 1984). The identification of 2,2'-diphenic acid in culture extracts indicates that *Polyporus* sp. S133 initiates its attack on phenanthrene through dioxigenation at C_9 and C_{10} to give *cis*-9,10-dihydrodiol. Dehydrogenation of phenanthrene-*cis*-9,10-dihydrodiol to produce the corresponding diol, followed by ortho-cleavage of the oxygenated ring, would yield the identified 2,2'-diphenic acid via 9,10-phenanthrene quinone. The observation of phthalic acid in the culture extract suggests that further degradation of 2,2'-diphenic acid proceeds via the phthalic acid pathway. Products of known metabolic pathways of phthalic acid through protocatechuic acid enter the central process of metabolism as pyruvate or succinate by *meta*- and *ortho*-cleavage of protocatechuic acid, respectively.

3 Conclusions

Phenanthrene, a PAH, was degraded by *Polyporus* sp. S133 in a MSB liquid culture. The maximum degradation rate (92%) was obtained when *Polyporus* sp. S133 was incubated for 30 days with agitation at 120 r/min. Furthermore, the degradation rate was affected by agitation and the kind of surfactant added. The mechanism of degradation was determined through the identification of several metabolites, 9,10-phenanthrene quinone, 2,2'-diphenic acid, phthalic acid, and protocatechuic acid. The capability of *Polyporus* sp. S133 to degrade phenanthrene can be used for the bioremediation of PAH-contaminated environments. Further study on the potential of *Polyporus* sp. S133 to degrade various other compounds is needed.

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